Thrombin generation

20. Thrombin generation, an essential step in haemostasis and thrombosis

H. C. Hemker

Thrombin (EC3.4.21.5) is the central bioregulatory enzyme in haemostasis. If functions at the level of the plasma, the platelet and the vessel wall and has also some actions less obviously related to haemostasis. In this chapter we will do two things: delineate its role in haemostasis and thrombosis and describe the mechanism of thrombin formation. Doing so we will necessarily touch upon part of the material that is treated in more detail in other chapters. Yet the detailed discussion of the individual factors, reactions and diseases may not automatically lead to recognition of the organised interplay between the elements of this biochemical system in which thrombin plays the pivotal role. Positive and negative feedback reactions, the cooperation between platelets and soluble factors, flow and surfaces, together govern thrombin formation at the site of a wound or a thrombus. We will try to give a picture of this system as a whole and to define its place in haemostatic and thrombotic events. We refer to the specific chapters for the many details that we must necessarily mention only in passing.

Traditionally the haemostatic process is divided into three stages:

1. Formation of the primary platelet plug: the reaction of platelets with the subendothelial interstitium makes them form an unstable plug
2. Stabilization of the primary plug: thrombin converts fibrinogen to fibrin, which cements together the loose plug and seals the vessel
3. Fibrinolysis: plasmin dissolves the fibrin strands in the course of wound healing

Often the recognition of these stages is the basis of a simplified concept in which primary haemostasis comprises all the platelet reactions whereas secondary haemostasis is identified with coagulation. By extension this leads to the concept of an arterial thrombus being a kind of primary plug that arises by platelet action only, without involvement of the clotting system whereas a venous thrombus would resemble a blood clot. The idea, popular in the 1960s and 1970s, that arterial thrombosis should be sensitive to antiplatelet drugs only and insensitive to anticoagulants is easily grafted upon this misconception. The importance of thrombin generation was maintained by some (e.g. Hemker 1975), but only in 1980 when the group of Loeliger published the sixty-plus reinfarction study was a change in opinion was initiated (see Meade 1984). Now we witness an increasing interest in thrombin-mediated reactions and their role in haemostasis and thrombosis. A posteriori, it seems only natural to recognise that thrombin generation, platelet reactions and vessel reactions cooperate closely in all forms of haemostasis and thrombosis. Thrombin is the most potent platelet activator and activated platelets are crucial in causing explosive thrombin generation. The components of the haemostatic mechanism do not operate in a sequence of solo performances but rather in concert with each other, each mechanism alternately taking the front but never without the accompaniment of the others.

THE ROLES OF THROMBIN

Thrombin and thrombosis

The two types of anticoagulant treatment, heparin and oral anticoagulation, are so different in their mode of action as well as in their side-effects that it can be safely assumed that where they share an effect, this is because of the only known property that they have in common: diminution of the amount of thrombin that is generated in clotting plasma.

Many trials have shown that anticoagulation is an efficacious treatment in different types of venous thrombosis whereas antiplatelet agents are not or are hardly effective (Hirsh 1986). The more interesting question is whether anticoagulation inhibits arterial thrombosis, because, if it does, this shows that thrombin plays a role in the formation of a platelet thrombus.

The Dutch sixty-plus trial and the Norwegian WARIS study (Sixty-plus Reinfarction Study Research Group 1980, Smith et al 1990) showed that oral anticoagulation can prevent recurrent myocardial infarction. More

recently it has been shown that heparin treatment can do the same (Neri-Serneri et al 1990). Aspirin is also preventative but comparison of the figures suggests that it is not more effective than adequate anticoagulation (Peto et al 1988, Steering Committee of the Physicians Health Study Group 1989).

There seems to be no doubt that antiplatelet therapy such as aspirin reduces mortality in unstable angina (Lewis 1983, Cairns 1985, Kerins & FitzGerrald 1991) and in myocardial infarction (Antiplatelet Trialists Collaboration 1988) but heparin appears at least as effective (Theroux 1988, Risk Group 1990).

In experimental thrombosis both oral anticoagulation and heparin diminish venous as well as arterial thrombosis. Inauen et al (1990, 1991) performed experiments in which blood from human volunteers was passed over the subendothelium of rabbit aorta at different rates. At low (venous) flow rates, fibrin deposition is the predominant phenomenon. It is prevented in a dose-dependent manner by heparin and at low levels of oral anticoagulation ($\approx$ 50% inhibition at an INR[1] of $\approx$ 2). At high (arterial) flow rates, platelet deposition is the predominant phenomenon. It can be inhibited by intense oral anticoagulation (INR 5.2) but is barely affected by heparin. Hirudin however inhibits experimental thrombosis in pigs under circumstances where heparin is ineffective (Badimon et al 1991). On the whole, experimental arterial thrombosis responds well to thrombin inhibition (Steel et al 1985, Badimon et al 1986, 1987, 1988, 1991, Lam et al 1986, Chesebro et al 1987, Heras et al 1988). It has also been demonstrated that, in an arterial thrombosis model, platelet-dependent thrombosis can be prevented by thrombin inhibition with synthetic thrombin inhibitors, hirudin and different types of heparin (Cadroy et al 1989) but antibodies against the GPIIb/IIIa complex of the platelet membrane are also effective, as is aspirin (Hanson & Harker 1988, Hanson et al 1988, Gruber et al 1989).

Suggestive evidence for a role of thrombin generation in the genesis of coronary infarction has been obtained by Meade et al (1986). They showed that an increase of the clotting factors I, VII and VII predicts a first coronary infarction with even more certainty than the cholesterol level does. Factors I and VIII are acute phase proteins that might rise with any type of illness or stress and thus may be simply accompanying phenomena without causal relationship with the thrombosis that is to come. For factor VII it is much more difficult to exclude a causal relationship.

One cannot escape the conclusion that in the generation of the arterial thrombus, both in humans and in experimental animals, thrombin plays as important a role as platelets do. This conclusion is only in apparent contradiction with the observation that platelet hyperreactivity, as observed by spontaneous aggregation in vitro, is a useful biological marker for the prediction of coronary events (Trip et al 1990). Platelet reactivity may be a step in thrombotic events that is harmless if not followed by thrombin formation, or thrombin formation may be a step in the mechanism of spontaneous aggregation, even in anticoagulated blood (see p. ●●●).

Thrombin and the bleeding time

Haemophiliacs have a bleeding tendency but their primary bleeding time is generally considered to be normal. Thrombopenias and thrombopathies, on the contrary, show a markedly prolonged bleeding time. This has led to the generally accepted idea that the bleeding time is determined by the efficacy of primary plug formation and therefore is a coagulation-independent phenomenon. There are reports however that deficiencies in factors I, V and VII cause a slightly prolonged bleeding time (Eyster et al 1981). Secondary bleeding, i.e. the bleeding of a reopened wound (Borchgrevink & Waller 1958), is prolonged in haemophilia and clearly dependent upon thrombin formation. The analogy with arterial and venous thrombosis is obvious, but the issue is more difficult to solve. Bleeding time determinations are notoriously imprecise and slight prolongations are difficult to demonstrate with the required statistical significance. Also it is not immediately clear whether a given experimental model represents primary or secondary haemostasis. In haemophiliac dogs the cuticula bleeding time is prolonged (Giles et al 1982), but it can be argued that the cuticula bleeding time is not a test of primary haemostasis. This may develop into a circular argument in which those types of bleeding time that can be shown to be influenced by clotting disorders are by definition deemed to measure secondary haemostasis.

If thrombin does play a role in primary haemostasis, should all bleeding times be prolonged when the clotting mechanism is disturbed? At first sight one is tempted to answer in the affirmative. If however primary plug formation was extraordinarily sensitive to thrombin, so that only traces of thrombin are required for its normal development, then, paradoxically, this very high sensitivity would lead to the same observations as in very low sensitivity. This is because thrombin generation so deficient as to not yield the necessary thrombin might not be compatible with life. The extreme sensitivity of platelets to thrombin cannot be doubted (see p. ●●●). Life-compatible coagulation disorders and antithrombotic treatment never induce zero thrombin generation. Clinical observations on clotting factor deficiencies and bleeding time therefore cannot be expected to solve the issue conclusively.

[1] NR = International Normalized Ratio = The ratio of the prothrombin time of the patient over that of a normal control plasma corrected in such a way as to make it independent of the type of thromboplastin used

Different alterations of the clotting system are reported to affect the bleeding time. A series of different heparins caused prolongation of the bleeding time in the rabbit that was strongly linked to the thrombin inhibition observed (Palm et al 1990). The often observed small local haemorrhage at the site of a subcutaneous heparin injection also indicates that a high local concentration of heparin under certain conditions may impair haemostasis in a small superficial wound.

Antibodies that inhibit the catalytic centre of thrombin induce a severe haemorrhagic syndrome (Scully et al 1982, Sie et al 1991), whereas an autoantibody directed against a secondary binding site that impaired interaction with fibrinogen, thrombomodulin and HC II, was even accompanied by a thrombotic tendency (Costa et al 1992). Hirudin, another thrombin-specific drug may cause prolonged bleeding times when given in high doses (Kaiser & Markwardt 1986, Markwardt et al 1988, Märki & Wallis 1990).

From these data it seems appropriate to conclude that thrombin plays a role in the early reactions of haemostasis. Observations on the (primary) bleeding time do not preclude, but on the contrary are rather suggestive of, a role of thrombin in primary haemostasis.

Thrombin and haemostatic plug formation

A series of morphological observations on the formation of the haemostatic plug gives a fairly detailed picture of this process and allows an assessment of the possible role of thrombin (Hovig et al 1967, Wester et al 1979, Giles et al 1982, Sixma & van den Berg 1984, Vander Velden & Giles 1988, Hong yu Ni & Giles 1992).

The first phase of the haemostatic reaction is the adhesion of platelets to collagen and other components of the subendothelial extracellular matrix, followed by their spreading on the surface. This reaction is thrombin independent. Platelets adhering to collagen will encounter the first traces of thrombin, maybe directly from the perivascular tissue. Collagen and thrombin are the primary, exogenous platelet activators, that together make platelets produce secondary platelet activators. Collagen and thrombin are also the only agents that induce the 'flip-flop' reaction and shedding of microvesicles in the platelet membrane, which provide procoagulant phospholipids and in this way foster thrombin formation. (For a review see Shroit & Zwaal 1991.)

A mass of platelets accumulates on the adherent platelets, by platelet–platelet interaction and thus plugs the bleeding vessel. Plasma is trapped in the interstices of the platelet mass. The abundant amounts of procoagulant phospholipids and the factor V released by the platelets produce the conditions ideally suited for massive thrombin generation. For platelet cohesion, it is required that fibrinogen is present and that the fibrinogen receptor forms by a combination of the glycoproteins IIb and IIIa (GP IIb-IIIa). The receptor forms reversibly when platelets are activated by secondary activators but irreversibly when they are activated by thrombin (van Willigen et al 1991).

In the platelet mass, no fibrin is observed, but morphological changes occur that are dependent upon thrombin formation and that are absent in the case of haemophilia and much retarded in a case of factor VII deficiency (VanderVelden & Giles 1988). These changes are interdigitation of the platelets, decrease of the intermembrane distances and widening of the surface connected tubular system. Only platelet plugs in which these phenomena are observed are stable. They are not seen in plugs in haemophilic dogs, but upon infusion of factor Xa and procoagulant phospholipids these processes become normal (Hong yu Ni & Giles 1992). Around the primary plug, fibrin strands are observed almost immediately (Wester et al 1979). In the absence of adequate thrombin generation the primary plug is unstable. These results indicate that thrombin is necessary for adequate primary plug formation by a mechanism independent of the formation of visible fibrin.

Fibrous transformation is the last phase of plug development. The fibrin clot at the outside of the aggregates extends inwards and gradually replaces the platelet mass. Fibrin threads that connect coherent platelet masses retract and serum is expelled. This process obviously is also thrombin dependent.

From these observations it follows that thrombin plays an important role in the formation of the primary haemostatic plug.

Thrombin and platelets

Recent, extremely elegant work by Vu et al (1991) has resolved the mechanism of thrombin activation of platelets. Thrombin splits off the N-terminal portion of a transmembrane molecule ('tethered-ligand activation'). The new N-terminal than acts as a ligand to the remainder of this protein receptor, which induces the activation signal. One of the reactions provoked is the membrane 'flip-flop'. In platelets, like in all other cells, the procoagulant phospholipid phosphatidyl serine (PS) is almost entirely located at the inside of the plasma membrane. That is why intact cells are not procoagulant whereas broken, lysed or otherwise damaged cells are. The platelet is the exception in that thrombin and collagen will induce a transbilayer movement of PS in the platelet so as to make the exterior of the intact activated platelet procoagulant (Bevers et al 1982). At normal platelet concentrations in the plasma, this mechanism can be provoked by thrombin alone and collagen has no additive effect (Béguin et al 1989). Platelets that are loaded with the Fura-2, so that the intracellular Ca^{2+} concentration and hence early activation

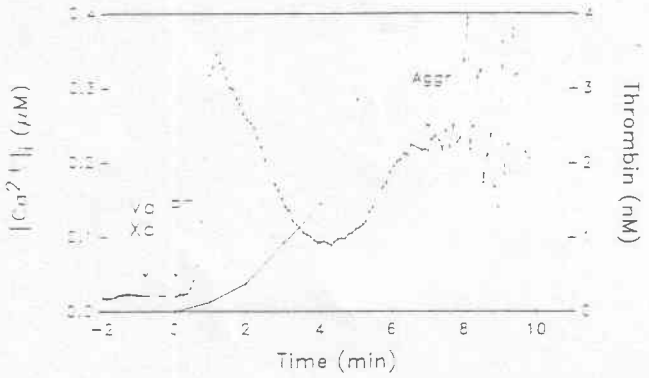

Fig. 20.1 Activation of prothrombin and platelets. Platelets (2×10^8/ml) loaded with the Ca^{2+}-indicator, Fura-2, were incubated at 37°C with factor Va (400 pM) and factor X_a (8 pM). At zero-time prothrombin (500 μM final concentration) was added. The increase in cytosolic Ca^{2+} (dashed line) was monitored by ratio fluorimetry (Heemskerk et al 1992a). At one minute intervals, the thrombin concentration was measured by subsampling into a solution of S 2238 (continuous line).

can be monitored, show activation when 0.1–0.2 nM of thrombin is generated in the medium (Fig. 20.1) (Heemskerk et al 1992b). This represents an extremely low thrombin concentration, if one considers that plasma contains ≈ 2 μM prothrombin, and that the concentration of thrombin reached in clotting plasma readily attains peaks of 200 nM (Béguin et al 1988).

Thrombin and the endothelium

Endothelial cells prevent thrombosis by both thrombin-independent and thrombin-dependent mechanisms:

1. Synthesizing and releasing the platelet inhibitor prostacyclin (PGI_2) and nitric oxide (Moncada et al 1976). This function is stimulated by thrombin which may be another example of a negative feedback action on thrombin formation by intact endothelium.
2. Providing thrombomodulin that binds thrombin with high affinity and makes it loose its procoagulant properties while it acquires the property to activate protein C (Comp et al 1982, Owen 1982).
3. Binding antithrombin III (AT III) via a proteoglycan of the endothelial plasma membrane and thus enhancing its activity so that it can more efficiently inactivate thrombin (Hatton et al 1978, Busch & Owen 1982, Colburn & Buonassissi 1982, Bauer et al 1983, Marcum et al 1984, Halton & Moar 1985, Shimada & Ozawa 1985).
4. Initiating the active internalization of thrombin (Lollar et al 1980a,b, Savion et al 1981, Carlson 1986).
5. Maintaining an asymmetry of the membrane that prevents procoagulant phospholipid species appearing at the luminal side of the cell (Shroit & Zwaal 1991).

Thrombin also may induce shape changes in endothelium, which exposes subendothelial matrix with tissue factor activity (Weiss et al 1989). However, it is questioned whether this phenomenon occurs in vivo (Thomas & Merton 1982).

Extravascular thrombin?

The proenzymes and inhibitors of the coagulation mechanism have a M_r less than 70 000, so they will probably occur in the extracellular fluid. The cofactors V and VIII have a M_r greater than 300 000 and will not normally occur extravasculary. Because tissue thromboplastin is present extravasculary on cells around the vessels (Weiss et al 1989) the conditions will allow a slow generation of thrombin extravasculary. Because of the presence of inhibitors, this will translate into a steady state of low thrombin concentration. Extravascular thrombin to our knowledge has never been directly demonstrated in healthy tissues. Fibrin deposits in inflammation and other conditions demonstrate that, under pathological circumstances, thrombin must occur commonly in the extravascular space. If it exists in normal tissues then a number of common enigmas will disappear; especially where positive feedback mechanisms require the presence of minute amounts of thrombin before the system can produce more.

Non-haemostatic activities of thrombin

Many actions of thrombin have been reported that are not immediately related to the haemostatic process, although sometimes one can imagine a role in tissue repair. Cell proliferation activity has been described for fibroblasts, splenocytes and endothelial tissue (Chen & Buchanan 1975, Teng & Chen 1975, Pohjanpleto 1977, Zetter et al 1977, Baker et al 1979). Other actions are chemotactic activity (see p. •••), stimulation of nerve cells (Sinder et al 1984); contraction of smooth muscle tissue (Walz et al 1985); initiation of bone resorption (Gustafson & Lerner 1983) and enhancement of viral infectivity (Dubovi et al 1983).

Thrombin interacts with almost all cells via membrane receptors (Shuman 1986). This interaction may be enzymatic, as in the case of the platelet receptor described by Vu et al (1991), but in other cases, such as when inactivated thrombin also produces the effect, one may guess that nonenzymatic binding of thrombin to the receptor causes the response.

The chemotactic activity is nonenzymatic and resides in a unique protein domain (prothrombin 338–400), possibly involving the anionic binding site of thrombin (Bar-Shavit et al. 1983, 1984, 1985).

α-Thrombin stimulates fibroblast receptors by two mechanisms; a nonenzymatic one mediated by α-thrombin, active or inactive, but not by γ-thrombin and a presumably enzymatic one, in which α- and γ-thrombin act, but

inactivated thrombin does not. Both mechanisms are necessary for inducing mitogenesis (Carney et al 1984, 1986).

Some of the actions of thrombin on cells may be via splitting of fibronectin or laminin (Goldfarb & Liotta 1986, Mosher & Thompson 1986), possibly acting also on laminin incorporated in a clot (Bale et al 1985). Thrombin binds covalently to platelet thrombospondin (Yeo & Detwiler 1985).

Thrombin increases the permeability of the endothelial cell layer by inducing a shape change that leads to the formation of gaps (Malik et al 1986). It also increases neutrophil adherence via an endothelial cell-dependent mechanism (Zimmerman et al 1986).

Thrombin-elicited aortic contractions are dependent upon a catalytically active enzyme and are evoked by physiologically relevant concentrations; 200 nM thrombin, a normal value attained locally in clotting plasma, is a sublethal dose when injected and causes maximal contraction of aorta smooth muscle cells (Walz et al 1985). Contraction is not dependent upon endothelium, but is probably dependent upon Ca^{2+} mobilization. Inactive enzymes do not interfere with the action of active thrombin, γ-thrombin is as active as α-thrombin. (See also: White et al 1975, 1980, 1984, Haver & Namm 1983, 1984.)

THE FORMATION AND INACTIVATION OF THROMBIN IN PLASMA

The core of the mechanism

The main chain of tissue thromboplastin-induced proteolytic activations in blood is:

$$VIII \Rightarrow X \Rightarrow II$$ [2]

For intrinsic coagulation the main chain of activation is:

$$PK \Rightarrow XII \Rightarrow XI \Rightarrow IX \Rightarrow X \Rightarrow II$$

All the non-activated clotting factors participating in these chains are proenzymes of serine proteases, the activated enzymes consequently are serine proteases. Clotting factor activation is an extremely slow process unless a suitable interface and specific protein cofactors are present. A complex of FXa, FV, Ca^{2+} and phospholipid is necessary to acquire the normal reaction velocity (Papahadjopoulos & Hanahan 1964, Hemker et al 1967). The activation of prothrombin by factor Xa in free solution is at only 0.001% of the normal speed (Esmon et al 1974a,b, Downing et a 1975). Phospholipids diminish the K_m for prothrombin conversion about a hundred times, which means that the enzyme can be saturated with substrate at the normal plasma concentration of prothrombin (≈ 2 μM), whereas factor Va increases the turnover number (k_{cat}) about 1000-fold, i.e. the number of thrombin molecules produced by the enzyme at saturation with the substrate increases a 1000-fold (Nesheim et al 1979, Rosing et al 1979, 1980, Lindhout et al 1982, van Rijn et al 1984).

The factor X activating enzyme consists of factors VIII a and IXa and phospholipid, forming a complex completely analogous to the prothrombinase complex (Hemker & Kahn 1967, van Dieyen et al 1981, 1985). Apart from their kinetic effect on k_{cat}, the factors Va and VIIIa also serve to improve the binding of their respective enzymes (FXa and FIXa) to phospholipid (Vande Waart et al 1983, van Dieyen 1985).

Factor VII and tissue thromboplastin form a complex that is again comparable to prothrombinase (Jesty & Nemerson 1974, Silverberg et al 1977, Zur & Nemerson 1980, Nemerson 1983).

We can summarize the reactions of the classical coagulation pathways as follows:

Extrinsic pathway:

$$[VII, TF, PL] \Rightarrow [X, Va, PL] \Rightarrow II$$

Intrinsic pathway:

$$PK \Rightarrow XII \Rightarrow XI \Rightarrow [IX, VIIIa, PL] \Rightarrow [X, Va, PL] \Rightarrow II$$

The importance of the role of the contact activating system in normal haemostasis has not been unequivocally demonstrated. As long as coagulation is discussed in terms of the two merging pathways depicted above, the contact-triggered intrinsic pathway is the only possible way to account for a role of the antihaemophilic factors VIII and IX. Since it has been shown, however, that factor VII can activate factor IX, the role of the antihaemophilic factors in thromboplastin triggered coagulation became apparent (Josso & Prou-Wartelle 1965, Østerud & Rapaport 1977, Marlar & Griffin 1981, Ma Xi et al 1989). This means that factor X can be activated either directly by factor VII and tissue thromboplastin or indirectly by factor IXa (together with factor VIIIa) that in its turn has been activated by factor VII. It is easy to see that the function of this pathway will still be dependent upon the amount of thromboplastin available. The contribution of the direct, one-step action of factor VIIa on factor Xa formation will be roughly proportional to the concentration of thromboplastin but limited in time because of the effect of the tissue factor pathway inhibitor (TFPI) (p. ●●●).

The contribution via the pathway:

$$VII \Rightarrow IX \Rightarrow X$$

will be subject to the same inhibition, but the result will be that a definite amount of factor X activator remains available in the form of factor IXa and its cofactors.

[2] In this and subsequent reaction schemes, the arrow (⇒) represents proteolytic activation and not chemical conversion. Square brackets ([ ]) indicate enzymatically active complexes. PK = prekallikrein, TF = tissue factor, PL = phospholipid. Roman numerals indicate the factors. II = prothrombin, etc.

With the recognition of this loop, the earlier contradiction between the clinical importance of the anti-haemophillic factors (VIII and IX) and the relative lack of importance of the contact factors disappears. This means that tissue factor becomes the only important trigger of coagulation, as long as no foreign surfaces play a role.

The fact that factor XI deficiency can give rise to serious bleeding, although it may equally well pass unnoticed, can however not be readily explained by this reaction scheme. Two possible solutions present themselves: in the first place factor XI is a substrate for factor XIIa. The latter factor also triggers components of the fibrinolytic system. Factor XI, as a substrate, is necessarily a competitive inhibitor of the fibrinolytic activation. This might cause haemorrhage by hyperfibrinolysis. The fact that the type of haemorrhage seen in factor XI deficiency often shows the clinical characteristics of hyperfibrinolytic bleeding (Seligsohn 1992) suggests this mechanism. A second possibility is that factor XI may be activated by thrombin (Gailani 1991) and thus may form part of a hitherto unrecognised feedback loop. Clinical observations suggest strongly that bleeding in factor XI-deficient patients is (also) determined by unidentified factors different from the factor XI level (Ragni et al 1985).

Feedback activation of thrombin formation

It is essential for the correct function of the haemostatic mechanism that thrombin formation be precisely controlled. The strong flow at the site of a wound, generated by the blood pressure, means that thrombin that was formed slowly, even in massive quantities, would be washed away. This will not be the case if thrombin formation occurs explosively after a given lag time. The strongly nonlinear generation kinetics of thrombin formation is caused by feedback activation, i.e. activation of thrombin formation by thrombin itself. This mechanism as such was recognised more than 40 years ago (Ware & Seegers 1948, Alexander et al 1950, Biggs & Macfarlane 1953). Yet its importance for the understanding of the kinetics of thrombin generation and the influence of anticoagulant drugs seems to have been somewhat neglected. Recent studies on the mechanism of action of heparins have stressed its importance (Ofosu et al 1985, Hemker 1987, Béguin 1988, 1989).

Both factor VIII and factor V have to be activated before they can play their role as protein cofactors to the clotting enzymes (Rapaport et al 1963). Although factor Xa is an efficient activator of factor Va (Monkovitz & Tracy 1990) and of factor VIII, in plasma thrombin seems to be the only enzyme that brings about these activations (Pieters et al 1989). Under the influence of thrombin, platelets will shed factor V, which subsequently is activated by the same thrombin. It has been shown that this activation rather than the release reaction itself is the rate-limiting factor for the generation of factor V activity from activated platelets (Baruch et al 1986).

A second procoagulant function of platelets, induced by thrombin as well as by collagen, is the platelet 'flip-flop' reaction (Shroit & Zwaal 1991). At the platelet concentration of normal platelet-rich plasma, thrombin alone is capable of inducing the flip-flop reaction, and platelet-mediated phospholipid availability is an important, rate-limiting, feedback process in thrombin generation.

The feedback activation of factor VII by its products, factor Xa, and factor IXa is discussed below as part of the starting mechanism of coagulation (p. ●●●).

Feedback inactivation of thrombin formation

It is equally essential that both the explosive formation of thrombin occurs and that it is limited to the site of the trigger, i.e. to the site of damage. Otherwise generalized thrombosis would be the logical consequence of the smallest wound. The limitation of the explosion is due to inhibition of the active complexes. This inhibition is sometimes triggered by the activated products (factor Xa and thrombin). This means that besides feedback activation, feedback inhibition also plays an important role in the coagulation mechanism. There are two important mechanisms that depend on the previous activation of a clotting factor and that lead to eventual inhibition of thrombin formation. These are :

1. Protein C- and S-dependent inactivation of factors Va and VIIIa
2. The tissue factor pathway inhibitor mechanism (tissue factor pathway inhibitor (TFPI) = extrinsic pathway inhibitor (EPI) = lipoprotein-associated coagulation inhibitor (LACI)).

Downstream of a site of damage in the vessel wall there will be a region with intact endothelium. Thrombin binds with high affinity to thrombomodulin and undergoes a modulation of its specificity. It is no longer capable of any action on the clotting factors I, V, VIII, or XIII. Instead it becomes capable of activating protein C. Protein C is a vitamin K-dependent pre-serine protease that, once it is activated by the thrombin-thrombomodulin complex, becomes a potent inactivator of factors Va and VIIIa. This means that the survival time of any prothrombinase and tenase action in a region lined by intact endothelium must be very short. The action of activated protein C is markedly enhanced by another vitamin K-dependent factor, protein S.

The tissue factor pathway inhibitor was recognised as early as 1952 (Hjort 1952), and rediscovered in the late 1980s (Rao & Rapaport 1987, Sandset et al 1987, Broze et al 1988, for a review see Sandset & Abildgaard 1991). It is a circulating plasma protein that is also adsorbed on the endothelial wall but can be released under the influence of

heparin (Sandset et al 1988). It has the capacity to bind to activated factor X and form a complex that is a strong inhibitor of the thromboplastin-factor VIIa complex. This mechanism ensures that tissue factor-induced factor X activation will stop as soon as a sufficient amount of factor X_a has been produced.

Platelets and thrombin formation

Platelets contribute to thrombin formation in four ways:

1. By the flip-flop reaction
2. By shedding phospholipid-rich microparticles
3. By releasing factor V
4. By creating a zone of zero flow within the platelet aggregate.

The flip-flop reaction is discussed in more detail above (see p. ●●●). The microparticles ('platelet dust') are small parts of the plasma membrane, pinched off during shape-change and flip-flop (Sandberg et al 1982, Shroit & Zwaal 1991). When thrombin generation is followed in platelet-rich plasma, a clot forms in the early phase of explosive thrombin formation. If this clot is removed, and with it the platelets, thrombin generation continues undisturbed, indicating that the phospholipid shed from the platelets can support normal thrombin generation (Béguin et al 1989).

The concentration of factor V in platelet-poor plasma is about 25 nM whereas that of its partner, factor X is around 200 nM. This may lead one to think that the contribution of platelet factor V may be important in vivo. The aggregation of platelets at sites where the haemostatic mechanism is active will cause a further increase in the ratio of platelet-factor V to plasma-factor V (see earlier). Factor V is released from platelets in its non activated form and the conversion into factor Va is rate-limiting for its activity. In plasma, factor V is present in a concentration (~ 25 nM) that is much lower than that of its partner factor X (~ 180 nM). The platelets from normal plasma-rich plasma will, upon complete release, double the plasma concentration of factor V. In a platelet aggregate the increase will be still more important.

It is often not recognised that thrombin formation in vivo will hardly ever occur in plasma as such, but always in or near platelet aggregates. The natural milieu for thrombin generation is probably the crevices within a platelet aggregate and in the tissue around a damaged vessel. In the bloodsteam, outside the aggregate, unless the blood is stagnant, flow will quickly dilute any activated clotting factor. If we take normal circulation time to be 40 seconds and if we assume complete mixing of the blood during this time, than it is clear that any local process has either to be extremely quick, as platelet activation is but thrombin formation is not, or has to be localized by stagnation of the flow. In normal haemostasis and arterial thrombosis the crevices of the aggregate are suitable candidates for the creation of microscopic stagnant pools. Stagnant pools of blood in a wound or in the venous circulation are obvious other possibilities.

The starting mechanism, partial coagulation mechanisms

The current views on the starting mechanism of coagulation, i.e. factor VII-tissue factor interaction, differ. One school holds that the proenzyme factor VII has a non-negligible enzymatic activity (Fujikawa et al 1974, Kisiel & Davie 1975, Nemerson 1983). Once factor VII(a) adsorbs onto tissue thromboplastin, its activity is enhanced so as to become sufficiently important to start the clotting process. An alternative view is that the activation of factor VII to its two-chain form is essential for its activity. This form can be generated from the one-chain form in a number of different ways, such as by activated factors X or IX (Radcliffe & Nemerson 1975, 1976, Seligsohn et al 1979, Masys et al 1982, Rao et al 1985, 1986). It is important to note that here again a feedback mechanism is operative: The products of factor VII(a)-tissue factor interaction, i.e. factors Xa and IXa, activate factor VII. The contact activation mechanism can also enhance factor VII activity in vitro (Altman & Hemker 1967); the cold activation of factor VII, involving kallikrein and different other proteins, also has been well established (Gjonnaes et al 1972, Gjonnaes 1973, Laake et al 1973, Laake & Ellingsen 1974, Czendlik et al 1985, Muller et al 1986).

The full coagulation mechanism discussed so far cannot be operative from time zero because blood, immediately after the coagulation reaction has been triggered, does not contain factor Va, factor VIIIa and sufficient procoagulant phospholipids. These have first to be provided by thrombin-dependent feedback reactions. So in order to start full-blown thrombin formation, incomplete partial mechanisms have to form small amounts of thrombin first.

When blood comes into contact with tissue thromboplastin (e.g. in the determination of the prothrombin time (PT) and probably in all haemostatic and thrombotic reactions in vivo), one can imagine factor VII (or factor VIIa activated in an, until now, unknown way) to activate some factor X. The factor Xa then, with the phospholipids available, may form some thrombin from prothrombin. These first molecules may start the feedback-activated full mechanism that is discussed above. When blood is activated via the contact activation system, as in the activated partial thromboplastin time (APTT), than activated factor IX, without factor VIIIa, will have to activate some factor X. This factor Xa, without factor Va will have to activate some prothrombin before the full mechanism can start.

In order for the full mechanism of coagulation to be operative, thrombin first has to be formed by a partial mechanism. We therefore cannot speak of the mechanism of blood coagulation. In fact there must be a series of subsequent mechanisms. This is especially important to keep in mind when interpreting the results of PT and APTT. These tests measure the lag time of thrombin formation. Therefore they are more likely to reflect partial mechanisms rather than the full mechanism.

Thrombin, fibrinogen and fibrin

Thrombin converts fibrinogen to fibrin (see Ch. 21). Fibrin is not an inert lattice. About 40% of the thrombin generated in plasma is incorporated into the generated fibrin, where it is protected from inhibitors (Liu et al 1979, Wilner et al 1981, Francis et al 1983). Fibrin thus plays an important role in limiting the activity of thrombin. Patients with a congenitally abnormal fibrinogen that does not bind thrombin, show a thrombotic tendency. Fibrin-bound thrombin retains its enzymatic activity and cannot be inactivated by (heparin-) AT III. During fibrinolysis it is released and may cause rethrombosis. Factor XIIIa arises from its plasma proenzyme (factor XIII) by the action of thrombin (Lorand 1986). In plasma, factor XIII is bound to fibrinogen. It is activated by thrombin, but only in the presence of fibrin strands, i.e. when its substrate is already present.

Radiolabelled catalytically inactivated α-thrombin incorporates into forming clots like active thrombin, whereas γ-thrombin and the hirudin complex do not (Wilner et al 1981). This occurs via an anionic binding region (Berliner et al 1985). This same region appears to be important for the binding of thrombin to certain cell receptors (Carney et al 1984). Activated neutrophils release elastase that can degrade clots and set free α-thrombin. Elastase also converts α-thrombin into a γ-thrombin-like form that has no clotting activity, but may retain other biological activities (Brower et al 1986).

Forms of thrombin

In addition to α-thrombin, the form of thrombin that is the immediate end product of prothrombin activation, two further thrombin products can be identified, β-and γ-thrombin. These are degraded forms that may arise from autodigestion of a thrombin preparation. No physiological functions have been claimed for these degradation products.

Meizothrombin is a molecule with thrombin activity that arises when only one bond is split in prothrombin instead of two (see Ch. 18). Meizothrombin contains a functional active centre but also the N-terminal fragments 1 and 2, i.e. the domain with the carboxylated glutamic acid residues. It is questionable whether meizothrombin arises during the clotting of normal plasma (Tans et al 1991). It has been postulated however that meizothrombin can form at the endothelial surface and play a role there in the negative feedback reactions mediated by thrombin (Tijburg et al 1991).

Inactivation of thrombin

In plasma, thrombin and factor Xa are quickly inactivated by a number of antiproteases. Antithrombin III is the main one, contributing 64% to the total antithrombin activity. α_2-Macroglobulin is the next most important antithrombin, contributing 23%, and the remaining antiproteases, primarily α_1-antitrypsin, are responsible for the remaining 13% of antithrombin activity (Hemker et al 1986). Heparin cofactor II has no activity unless activated, e.g. by dermatan sulphate. The half life of thrombin in plasma is 14 seconds at an AT III concentration of 2 μM. The corresponding half life of factor Xa is 32 seconds. Active heparin with a chain length of 18 monosaccharide units or higher increases the activity of AT III both towards thrombin and towards factor Xa several 1000-fold. In plasma, the rate of inactivation of thrombin and factor Xa is proportional to both the AT III and the heparin concentration (Béguin et al 1992). The ratio of the inactivations remains always the same, i.e. thrombin is always inactivated about three times as fast as factor Xa, independent of the presence of heparin. Active heparins with a chain length of between five and 17 monosaccharide units are inactive towards thrombin inactivation but maintain their activity towards factor Xa inactivation. Factor IXa is insensitive to AT III action but not to the action of the AT III-heparin complex (Pieters et al 1990).

The effect of AT III, and hence of heparin on the appearance and disappearance of thrombin in clotting plasma is complex. Enhancement of thrombin inactivation has two effects. There is a direct and proportional effect on thrombin, which means that, when thrombin disappears twice as fast as normal because of the presence of heparin, then the concentrations of thrombin seen in clotting plasma are half normal. In addition there is an inhibition and retardation of thrombin generation velocity that is due to the inhibition of thrombin-dependent feedback effects. The extent of this inhibition is dependent upon the experimental circumstances. It is much more obvious in the intrinsic coagulation pathway than in the extrinsic one. This is why the APTT is much more sensitive to heparin than is the prothrombin time (PT) (Hemker 1987, Béguin et al 1988).

Factor Xa is present in excess when plasma clots after initiation by either the extrinsic or the intrinsic pathway. For the prothrombin converting activity, factor Va is the

rate-limiting factor when enough procoagulant phospholipids are present (PT and APTT). Phospholipids are rate-limiting for thrombin generation in platelet-rich plasma. When they are supplied, factor Va again becomes rate limiting (Béguin et al 1989). Because of the excess of factor Xa, inhibiton of factor Xa is an inefficient means of inhibiting thrombin generation. However, factor IXa inhibition contributes significantly to the inhibition of intrinsic thrombin generation.

The complex of thrombin and α_2-macroglobulin has no known activity on any high molecular weight (i.e. physiologically important) substrate. It maintains activity against small molecular weight, chromogenic substrates. This accounts for the considerable thrombin-like amidolytic activity in serum.

THE ASSESSMENT OF THROMBIN DIRECTED ANTITHROMBOTIC ACTION

We may conclude from the above that the enzymatic action of thrombin on a large number of substrates is essential in haemostasis and thrombosis. The amount of thrombin that can express itself in the process of haemostasis or thrombosis will therefore determine the magnitude of the haemostatic and thrombotic response. Evidently it is not important how the amount of thrombin is decreased. Normal prothrombin conversion, inhibition of the thrombin formed (hirudin, dermatan sulphate, most heparins), inhibited-prothrombin conversion and normal thrombin inactivation (oral anticoagulation, pentasaccharide) will all have antithrombotic and anti-haemostitic effects.

It would be useful to have a test that could be carried out in the plasma of a patient and that would directly indicate the antithrombotic effect obtained by thrombin inhibition. Current practice only provides indirect measures that may or may not correlate with the antithrombotic effect. The prothrombin time correlates with the antithrombotic effect brought about by oral anticoagulation. The APTT indicates the effect of heparin. Dermatan sulphate or pentasaccharide will hardly influence either PT or APTT. The fact that different drugs, at effective concentrations, have largely different influences on these tests already indicates that neither can be the universal parameter that we look for. The reason for this is easy to imagine. Clotting invariably occurs when a level of 10–20 nM of thrombin is reached in blood or plasma. That is shortly after the onset of the full coagulation mechanism. The clotting time therefore measures the lag-time of thrombin formation during which mainly partial clotting mechanisms and thrombin mediated feedback are operative. Consequently the influence of a drug on a clotting time largely represents its effect on a partial, initiating mechanism and not on the full mechanism. Yet it is the effect of these drugs on the full mechanism of thrombin generation and inactivation that determines the antithrombotic effect.

A simple clinical observation illustrates this point. A patient with a congenital AT III deficiency of 50% will have a thrombotic tendency. Yet none of the possible variants of the clotting time will show significant prolongation. Oral anticoagulation will bring the thrombosis risk to normal and at the same time decrease the PT and APTT values to pathologically low levels.

One entity that is changed in these patients is the amount of thrombin formed. More precisely: the amount of thrombin formed and the time that it has to act. Thrombin is an enzyme, and in which a fair approximation one can expect that 1 nM of an enzyme acting for 10 seconds will have the same effect as 10 nM acting for 1 seconds. This means that the product of time and concentration determines the effect of thrombin. A normal amount of thrombin that persists longer because of a congenital lack of antithrombin III may cause thrombosis. A tendency to thrombosis can be remedied by lowering the amount of thrombin formed via oral anticoagulation or by making a normal amount of thrombin disappear faster than normal via heparin administration (Fig. 20.2). This demonstrates that the product of concentration and time is an important variable. We called this variable the *thrombin potential* (*TP*) (Hemker et al 1989). It is not a new concept, the area under the thrombin generation curve, introduced over 40 years ago (Biggs & Macfarlane 1953) is precisely the same thing. The TP has the dimension of time multiplied by (thrombin) concentration and its value in normal plasma, under the experimental circumstances of Béguin et al (1988), is given in Table 20.1. The values of TP obtained in plasma may depend upon the experimental conditions, such as intrinsic or extrinsic clotting. They vary only a little with thromboplastin concentration.

It is claimed that there are antithrombotic drugs, like dermatan sulphate, pentosan polysulphate or lactobionic acid, that 'do not influence coagulation'. In our experience they have indeed little influence on the APTT but they invariably bring about a significant decrease of the TP.

The enzymatic action of thrombin means that the concept of TP can be used in yet another way. If we imagine a blood platelet that is being triggered by thrombin, then the triggering will take effect after a certain number of specific receptor molecules at the platelet surface have been split. This can be brought about by a high concentration of thrombin in a short time or by a lower concentration in a proportionally longer time. Again the product of time and concentration counts. The same holds true for the clotting of fibrinogen. Clotting will occur when a given concentration of fibrin monomers has been reached,

We developed a method to determine the TP in a

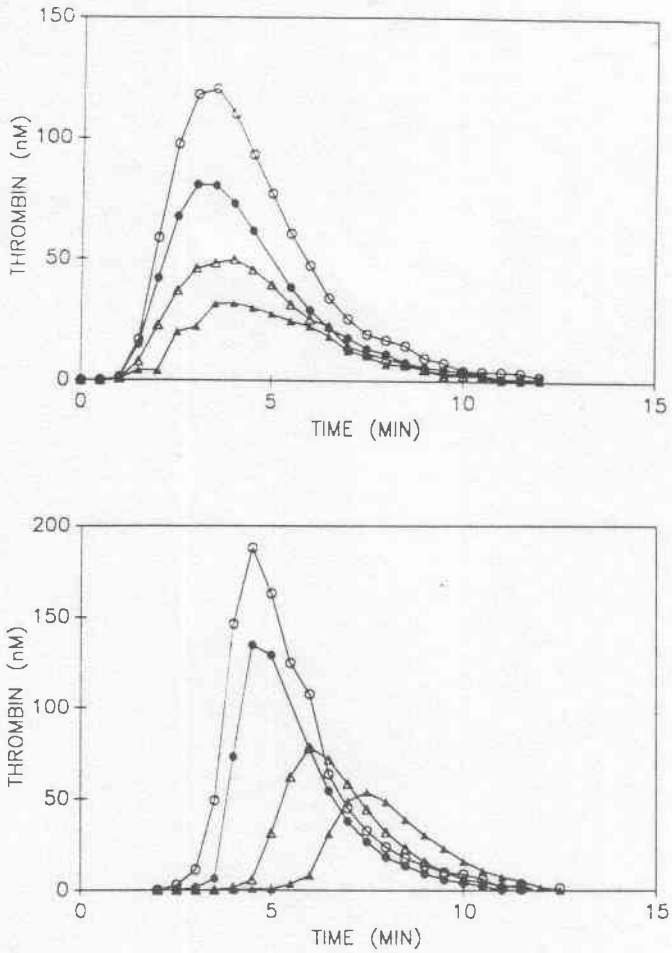

Fig. 20.2 Influence of heparin on thrombin generation. A Triggered with tissue thromboplastin B. Triggered via contact activation. It is seen that in the system comparable to the prothrombin time measurement (A), thrombin generation is inhibited to a comparable extent as in the APTT-like system (B). However in the latter there is a prolongation of the lag-phase of thrombin formation because of the necessary feedback activation of factor VIII by thrombin. ○, control; ▲, 0.5 μg/ml; ●, 1 μg/ml; △, 1.5 μg/ml of a heparin preparation. (From Béguin et al 1992.)

Table 20.1 Values of the endogenous thrombin potential Time (TP)

	TP (μM/sec)*
A. *Values obtained in plasma*	
Extrinsic system	29.2 ± 4.3
Intrinsic system	30.5 ± 5.3
B. *Threshold values*	
Clotting of normal plasma	1.1
Platelet aggregation	0.2
Ca^{2+} influx in platelets	< 0.005

* TP reflects the thrombin concentration and the time in which it acts

i.e. when a given amount of thrombin has been acting on the fibrinogen for a given time (Hemker et al 1979). In this way one can speak of a threshold value of the TP necessary to provoke a given phenomenon (Table 20.1).

In these terms one can think of thrombin-mediated antithrombotic medication as the administration of a drug so as to decrease the TP value to under the threshold limit of thrombogenesis while remaining above the threshold of haemostasis. It remains an open question whether the nature of the drug has an influence on the thresholds. Those who look for the ideal anticoagulant, that prevents thrombosis without influencing haemostasis tacitly assume that this is possible. In our opinion this is not very likely. The differences between drugs are due to their different pharmacokinetic properties, which affect the ease with which they can be maintained in the required range of plasma levels (e.g. low molecular weight heparins versus unfractionated heparin). Also secondary circumstances may intervene. Drugs that act via heparin cofactor II (dermatan sulphate, lactobionic acid) can hardly be overdosed because the plasma level of heparin cofactor II is lower than that of prothrombin, so that the TP cannot be inhibited beyond a certain level.

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We developed a method to determine the TP in a continuous procedure adaptable to the routine laboratory (Hemker et al 1993).